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Sustainable fermentation processing of two revalorized agro-industrial discards: carrot and brewer's yeast

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Abstract

An integrated and sustainable fermentation process was developed which enabled both the revalorization of two regional agro-industrial discards as well as by-product reuse. Carrot and brewer's yeast, which are commonly used for animal feed, were processed to obtain 77.5 L of ethanol, 450 kg of solid waste called bagasse, 970 L of liquid effluent called vinasse, and 39.8 kg CO₂ per each ton of discarded carrot. Results showed that the obtained bagasse was suitable for feeding 55 animals (calves). The dilution of vinasse with fresh water (1:5) satisfied the requirements necessary to be used as beverage for the same number of animals, leaving a remnant which could be newly diluted (1:5) and used to irrigate a 0.025-ha carrot crop, the land dimension required to grow 1 ton of carrot.

Keywords: Discards, Carrot, Yeast, Fermentation, Ethanol, Sustainable

Background

First-generation biofuels, derived from agricultural products (corn, sugarcane, palm oil, rapeseed, or soybean), have produced an impact on grain prices, tightening the supply chain and the availability of land for food production [1]. The transformation of forests, savannahs, or pastureland into farms for biofuel production could lead to greater CO₂ emission than the emission reduction these biofuels could produce [2,3]. Consequently, serious damages to the ecosystems have been produced. Second-generation bioethanol is a biofuel made from nonedible raw materials [4]. Wastes or discards should no longer be seen as a burden but rather as another source of material such as energy fuel [1,5]. Thus, some organic waste may be incorporated as low-cost raw materials for second-generation bioethanol production, depending on the waste composition. Due to the wide variety of technological alternatives for the production of ethanol through fermentation, a global analysis of the process is required,

leading to the development of a sustainable process, which cooperates with the final disposal of certain existing wastes in each region [6-9].

A particular case of a fermentable substrate is the one represented by the discards of carrot (*Daucus carota*) cultivation. The cultivated area in Santa Fe (Argentina) is approximately 1,500 ha, and its average yield is nearly 40 tons (t) ha⁻¹. Only 65% to 90% of carrot harvest meets the quality standards; consequently, during harvest time, 20 to 100 t of carrots with an optimal degree of freshness and maturity are discarded daily due to a sizing problem and then directed to animal feed [10]. Carrot is one of the most efficient crops in biomass accumulation [11], and it may be enzymatically hydrolyzed to increase yields [7]. In general, plants accumulate carbohydrates, e.g., starch. However, carrot is one of the few plants that accumulate free sugars into vacuoles (40% to 60% of total carbohydrates) as reserve carbohydrates. For free sugars, 95% is composed of sucrose, fructose, and glucose, which, together with terpenes, are the main determinants of carrot taste. Among the carbohydrates present in carrots, reducing sugars (RS) (fructose and glucose) are present in an equimolecular amount [12,13]. In addition, in the same geographical area, 1 t of *Saccharomyces cerevisiae* yeast cells is discarded daily by a beer manufacturing industry. So far, the elimination of these yeast

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cells has not been solved yet due to their elevated biochemical oxygen demand (BOD), and consequently, no process is applied to reuse them [10].

Taking into account the above considerations, the aims of this study are the following: (1) to develop a sustainable process for production of second-generation ethanol from two regional agro-industrial discards: carrot as substrate and brewer's yeast as biocatalyst, and (2) to qualitatively and quantitatively evaluate the effluents generated by the process in order to propose solutions to make it sustainable.

Methods

Raw material, handling, and storage

Discarded roots of carrot (DC) (*D. carota*) were collected in November to December from a packing shed in the Santa Fe area (31°25'S, 60°20'W), Argentina. These discards were by-products from carrot packing processes in which plant leaves were cut and whole roots were washed, dried, and selected before packing. DC were stored in shed under ambient conditions (20°C to 32°C, 50% to 60% humidity). During delivery of random representative samples obtained by sampling methods [10], carrots were packed in a polypropylene container and moved to the laboratory where they were used immediately.

Preparation and pretreatment of feedstock

Batches containing 0.5 kg of discarded carrot milled to a particle size less than 4 mm were dipped into water in a total volume of 1 L and mixed with the enzyme OptimaseCX255L (thermostable xylanase from *Trichoderma reesei*). The initial pH was then adjusted at 5.5, and the temperature was adjusted to 70°C, performing the hydrolysis during 2.5 h. After hydrolysis, they were compressed and filtered to obtain a liquid fraction and a solid residue. The pH of the liquid fraction was adjusted to 4.5 and was called carrot must (CM). The solid residue was called bagasse.

Microorganism

S. cerevisiae CCUB filtered and discarded by a local brewing industry after five operative cycles of use was obtained directly from filters. Whole yeast cells were kept in a sterile container, without nutrient addition, at 4°C and at saturation humidity for 4 days. For inoculum preparation, cells were suspended in an isotonic solution and counted by a differential direct-count method in order to calculate the volume required [14].

Fermentation conditions

Fresh must was used in every case, and its pH was adjusted to 4.5. The inoculums were adjusted to a value of 10^8 cell mL⁻¹. Batch fermentations were developed at 28°C in a 500-mL stirred tank bioreactor, equipped with

controlled heating and stirring systems. The agitation speed was regulated at 100 rpm in all of the experiments. The fermentation progress was monitored following CO₂ production, which was collected in a gasometrical probe. Samples at different reaction times were taken and submitted to centrifugation. Supernatants were stored and conserved at -20°C. All experiments were performed in triplicate [7].

Recycle of the biocatalyst

For this experiment, a reactor was required and equipped with a filter which has a 3-μm pore size at the exit of products and a pump that allows the injection of nitrogen gas to facilitate the filtration process during the product output. When fermentation finished, the reactor content was filtered, leaving the cells retained in the filter. The reactor was then refilled with fresh must and then subjected to further agitation (200 rpm) for 10 min so that the yeast retained in the filter was dispersed again, and the must temperature reached 30°C to start a new fermentation cycle.

Analytical methods

Moisture

The moisture of carrots was determined using an approved method (44-15A) [10].

Sugar concentration

The concentration of RS was measured using the 3,5-dinitrosalicylic acid method. The concentration of total sugars (TS) was assayed by the same method after acid hydrolysis (1.2 mol L⁻¹ HCl, at 65°C for 15 min), neutralization with 1 mol L⁻¹ NaOH, and filtration [15].

Ethanol concentration

Ethanol concentration was determined by GC (Sigma 3B, dual flame ionization detector (FID) chromatograph, PerkinElmer Instruments, Branford, CT, USA). A FID and a packed column of Chromosorb 102 (2.0 m in length) were employed. The column oven was operated isothermally at 150°C, and the injection and detector ports were kept at 195°C and 220°C, respectively. Nitrogen was used as carrier gas with a flow rate of 30 mL min⁻¹, and the combustion gas was a mixture of hydrogen and air. Iso-propanol (Anedra, Argentina, >99.9%) was used as internal standard [16]. In the chromatograph of every fermented sample, only two peaks appeared: one corresponding to the internal standard, and the other corresponding to ethanol.

Analysis of effluents

The analysis of effluents was performed according to the regulations applied in Argentina [17].

Parameters

Ethanol yield per carrot ($Y_{p/c}$) was considered as the ratio of total ethanol produced and used carrot (dry base) (g g^{-1}) [18]. Sodium adsorption ratio (SAR) index represents the sodium adsorption and is calculated by the ratio of the sodium concentration to the square root of the halved sum of calcium and magnesium concentrations [19].

Statistical analyses

In order to examine the reproducibility of the experiment, three experimental runs of each batch operation were conducted, and determinations were performed in duplicate. The results were expressed as the average value and its standard deviation. Data were analyzed utilizing the one-way ANOVA procedure of the SPSS software. Differences between means were detected by Duncan's multiple range test. Data were considered significantly different when $P < 0.05$.

Results and discussion

Integration of the three steps of a fermentative process

In order to build a sustainable process of ethanol production from discarded carrots and yeasts, an efficient extraction of carrot sugars during the preparation of must is essential as well as an adequate fermentation process, which requires low energy demand and low equipment investment [9]. In the pursuance of these objectives, the options for the three main stages of the process are discussed.

Preparation of must

During CM preparation, the enzymatic hydrolysis of carrot tissue allowed us to obtain a must with $150.2 \pm 0.6 \text{ g TS L}^{-1}$. This means that 151.7% of sugars were extracted, which could not have been achieved if the enzymatic hydrolysis of the tissues had not been carried out. The analysis of carbohydrate composition of the must showed that the sugars were sucrose, fructose, and glucose, all of them capable of being used by the yeast through alcoholic fermentation [7]. This stage left as discard 450 kg of bagasse from 1 t of processed DC as discard.

A viable alternative to favor hydrolysis during must preparation could be the optimization of a semicontinuous or continuous process with immobilized enzymes, which would decrease product inhibition occurring on the cellulases, thus decreasing their cost [20].

Fermentation

Fermentation of CM using free cells of *S. cerevisiae* CCUB discarded by a local beer industry allowed us to obtain an ethanol yield $Y_{p/c} = 0.403 \text{ g g}^{-1}$ and 39.8 kg CO_2 which was equimolarly generated. In order to evaluate the possibility of increasing the lifetime of the biocatalyst, reducing the use of yeast cells, and avoiding the generation of a

greater amount of residue, we studied the efficiency of the biocatalyst in consecutive fermentations, in which yeasts were recycled. Average concentrations of ethanol together with their standard deviations are shown in Figure 1 in which it can be observed that the ethanol yield remains constant during at least five recycles of cellular biomass, showing an increase in productivity (data not shown), since it reduces the lag phase time. Furthermore, it was observed that the carrot juice had no inhibitory effect on the strain of *S. cerevisiae* CCUB during this period. In addition to these results, further research is needed to assess the convenience of total, partial, or no replacement in order to ensure an appropriate inoculum, leaving the lysed cells as a nutrient of the must and increasing their nitrogen content.

Distillation

Finally, ethanol may be separated by simple distillation of the fermented must, leaving 970 L of a liquid effluent called vinasse per each ton of processed carrot. Subsequently, according to the use for which ethanol is intended, it may be subjected to various processes of purification and rectification. Thus, from the optimal integration of each stage, a yield of 77.5 L of second-generation bioethanol is obtained.

Characterization and disposal of process discards

The quantity and quality of discards generated by any process depend on the process conditions. Alternatives of reutilization of the bagasse and of the vinasse, depending on their chemical composition and their quantity, are shown. The remnant of yeasts and CO_2 are not analyzed in this work because the former are proposed to be recycled and the latter could be liberated to the atmosphere to be consumed by carrot plants during their growth.

Bagasse

This solid discard is composed of disintegrated carrot tissue, with an average moisture content of 67%. In a study where the primary focus was alternative food for the months of drought, Castillo and Gallardo [21] reported that carrot was classified as a voluminous nontraditional food that, due to its chemical composition (dry matter 7% to 30%, crude fiber 5% to 11%, and protein 4% to 16%), is considered a suitable food for incorporation into the diet of cattle, up to 30% in dry matter, without impairing the production of milk in cows. Furthermore, it is low in calcium and phosphorus, and its digestibility could be enhanced if it is combined with a high concentration of soluble carbohydrates (50% to 75%) as these cause high rates of ruminal fermentation. If necessary, the carrot content of proteins can be balanced with different sources of

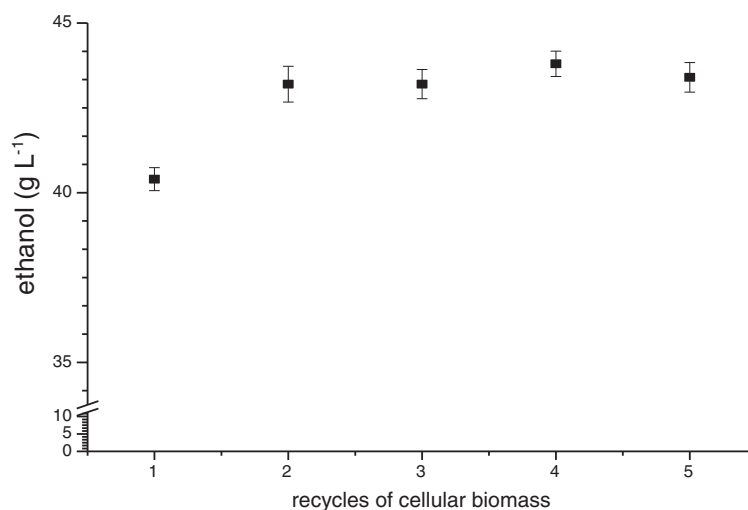


Figure 1 Ethanol concentration obtained through fermentation with recycles of cellular biomass.

nitrogen such as the yeasts used as biocatalysts in case they were discarded.

Taking into account the difficulties in bagasse transport, handling, and supply caused by its deterioration speed, this work proposes that bagasse be used to feed the animals in nearby fields, which are precisely the animals that are feeding on carrot discards today. Besides, the difficulties mentioned could be counteracted by keeping bagasse silage in combination with 15% to 25% of poultry farm waste [21]. Additionally, other alternatives for the reuse of the carrot bagasse could be its utilization as soil fertilizer or as substrate for composting.

Vinasse

Vinasse was characterized within the parameters established in law number 11220 of the Province of Santa Fe [17]. The results are shown in Table 1 (A) and are analyzed.

Taking into consideration the values of COD and BOD₅, it is clear that not only the organic load of the effluent is very high but also its biodegradability index (>0.5). Organic compounds could be reduced by applying a biological treatment such as the aerobic treatment of active mud or sewage if the final destination is a watercourse.

In this effluent, the remaining carbohydrates of the fermentation represent the most important source of organic load, and therefore, the control of this loss could reduce the environmental impact involving an increase in process efficiency and a significant reduction in the cost of final biological treatment. Furthermore, although the amount of fat detected in this effluent is very low due to the chemical composition of the substrate, its presence should be controlled so as not to influence on the effluent treatment.

Temperature control is very important because it influences on the rate of chemical and biochemical reactions, the solubility of gases and minerals, and the growth and respiration rates of aquatic organisms. The amber color, analyzed by sensory evaluation, is the consequence of the presence of beta-carotene; therefore, it is not expected to affect the passage of light.

In the same way, the sensory analysis of smell indicates that the slight yeasty odor is a result of the loss of biocatalyst in the last stage of filtering, while no harmful effects on the environment are produced. This should be a

Table 1 Characterization of liquid effluent according to law number 11220 of the Province of Santa Fe

Parameter	Value	Units
A Chemical oxygen demand (COD)	44,000	mg O ₂ L ⁻¹
Biochemical oxygen demand (BOD ₅)	22,750	mg O ₂ L ⁻¹
Total solid	22,600	mg L ⁻¹
Sedimentable solids (2 h)	<0.1	mL L ⁻¹
Fats	1.3	mg L ⁻¹
pH	4.4	
Color	Amber	
Smell	Slight	
Temperature	80	°C
Specific conductivity	9.68	dS m ⁻¹
Total hardness (Ca ²⁺ + Mg ²⁺)	700	mg CO ₃ Ca L ⁻¹
B Sodium	358.6	mg L ⁻¹
SAR index	18.9	SAR
Potassium	2,270	mg L ⁻¹
Nitrogen	1,481	mg L ⁻¹
Phosphorus	150	mg L ⁻¹
Sulfate	810	mg L ⁻¹

critical control point of the process so that the yeast content in the effluent can be minimized.

Of the inorganic compounds, calcium and magnesium cations, which are present in forming various types of salts, are the most important ones because they are responsible for the hardness of the waters. In addition, in relation to sodium, the SAR index was determined. The content of iron and remaining cations is generally not contemplated by the laws regulating effluent discharge. Nevertheless, the respective determinations shown in Table 1 (B) were performed in order to examine their nature and amount. The content of nitrogen and phosphorus is beneficial since they are known as the major nutrients or bio-stimulants of protists and plants so that, when they are not in the required proportions, they will be added to ensure proper biological treatment. Another important element is sulfur because it is required for protein synthesis. Toxic minerals like copper, lead, chromium, silver, arsenic, and boron were not analyzed since vinasse comes from the processing of carrot with water.

Therefore, in view of the composition of the liquid effluent of the process proposed (Table 1 (A)), it can be observed that in the event it is poured into a watercourse, it should be first cooled, neutralized, and biologically treated to reduce its organic load. However, its discharge would be subject to the dilution capacity of the receiving body and the final receiving body, as required by regional laws. Further research is needed to investigate whether the effluent liquid may be reused to be added to the enzymatic hydrolysis stage of must preparation. In this way, it may be possible to avoid the use of water to reduce the cost of correcting the pH, but the ionic strength must be taken into account because it could alter the activity of the

Table 2 Yield of crop in ethanol production

Crop	Agricultural yield (t ha ⁻¹)	Ethanol yield	
		(L t ⁻¹)	(L ha ⁻¹)
Sugarcane ^a	100	70	7,000
Corn ^a	8	370	2,960
Yucca ^a	20	180	3,600
Sweet sorghum ^a	35	86	3,010
Beet ^a	60	100	6,000
Carrot	40	53	2,120

^aAdapted from Sanchez and Cardona [2].

biocatalyst. However, emphasizing the parameters in Table 1 (B) and considering that the fat content and odor are suitable, two alternative uses for the liquid effluent were evaluated: as irrigation water or as beverage for livestock. The characteristics of each of these alternatives are discussed.

Irrigation water Three important requirements of moisture of the carrot crops support the proposal of using vinasse as irrigation water. A progressively irrigated crop produces carrots of higher caliber because both the photosynthetic activity and the rate of sugar accumulation increase. The cultivation of carrot requires neutral or slightly alkaline grounds and is also very sensitive to salinity, to specific conductance values greater than 2 dS m⁻¹ [22]. However, as disaggregated soils with not much organic matter are used, the salts present in irrigation decrease their importance as they do not accumulate in the soil.

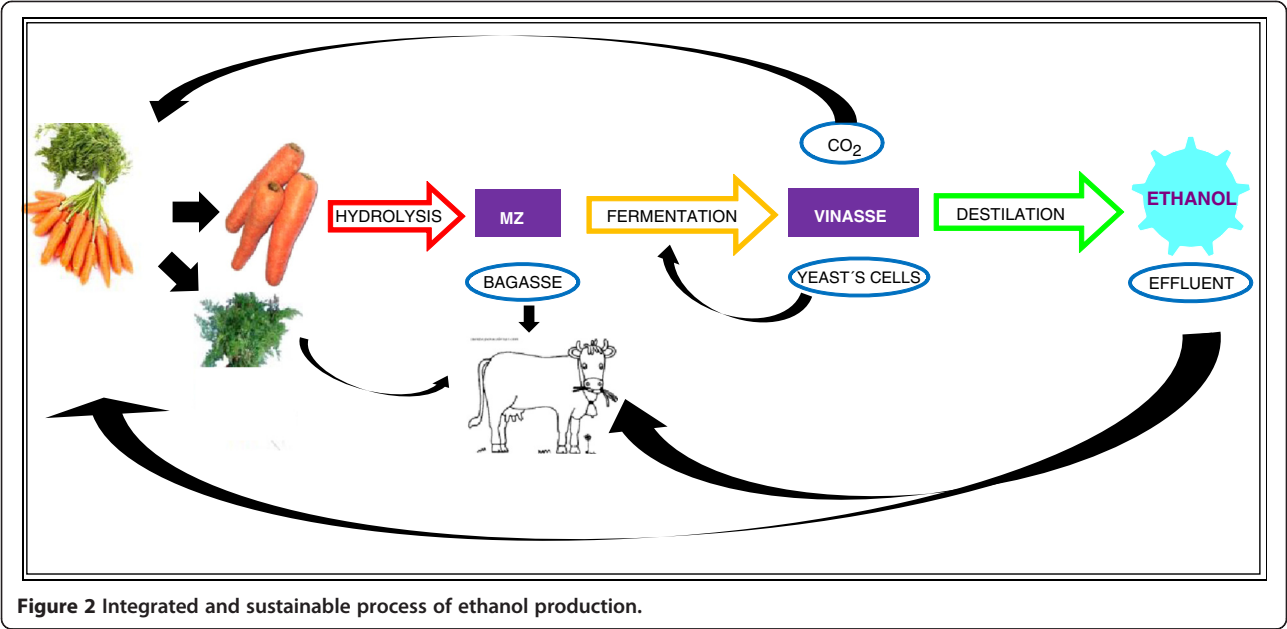


Figure 2 Integrated and sustainable process of ethanol production.

In this sense, the water quality indicator parameters developed by the consultative committee of the University of California and later extended by Ayers and Westcot [19] were analyzed. The combination of these parameters yields the degree of aptitude as irrigation water. According to these parameters, vinasse is defined as suitable for irrigation, with strict restrictions for its use due to its salinity, but there is no restriction if permeability is considered. In addition, it presents no toxic elements and has a good amount of bio-elements such as nitrogen, sulfur, and phosphorus, which are generally added as fertilizers to crops of vegetables like carrots. Since approximately 16 g L⁻¹ of total solids are sugars, it is not convenient to use it directly for irrigation because it may stimulate the proliferation of some microorganisms and cause a mismatch in the soil microflora. A previous biological treatment is necessary after reducing its temperature.

As a consequence, the large amount of sodium and potassium ions could not affect the crop if the effluent is diluted to 1:10, which could be achieved by a combination with the rest of the water normally used for crop irrigation so that the pH would be consequently adjusted under these conditions. Therefore, water for irrigation would be obtained as enriched with nitrogen, phosphorus, potassium, calcium, and magnesium, which are the most recommended fertilizers for crops with good yields [22,23].

The volume obtained by diluting the liquid effluent produced by processing 1 t of carrot exceeds the requirement of water necessary to irrigate 0,025 ha of cultivation, which is the area required to harvest this amount (yield 40 t ha⁻¹), so it is necessary to explore some other application of the effluent in order to make the process sustainable.

Beverage for animals This alternative is based on the additional benefits that arise when using a beverage rich in sugar for animals that were fed with bagasse, improving digestibility while increasing the content of dietary phosphorus [21]. At this point, it is important to note that the liquid effluent can be used as a beverage for animals without submitting it to any prior biological treatment but requires a dilution of approximately 1:5 so that the content of sodium and potassium would not affect the health of livestock [24].

Design of an integrated and sustainable process

The integration of each stage allowed us to revalue one of the quantitatively most important agro-industrial discards of our region. Thus, it is possible to return to the soil and the ecosystem the majority of the elements extracted by carrots during their growth, making it a sustainable promising process for obtaining second-generation biofuel. As it can be observed in Figure 2, after harvesting 1 t of carrots, leaves were removed and used, together with the

bagasse produced during CM preparation, to feed livestock. CM is then fermented in a batch process with biomass recycling from which fermented must and CO₂ are obtained, with the latter returning to the atmosphere. The yeast-free cells are reused in the same process or are employed for cattle feed if in excess. By distilling the fermented must, ethanol is separated and vinasse can be first diluted to 1:5 with water to reduce its salt content and correct its temperature and pH (which must be greater than 5.5). This gives a volume of about 4,850 L, of which 50% is required as a beverage for the same number of calfs, while the rest can be diluted again to 1:5 and used as irrigation of 0.025 ha of carrot crop.

To carry out this process, we propose the construction of a mobile module in which carrot processing operations take place: hydrolysis, fermentation, and distillation. This mobile module could be taken to the different packing shelves where carrot discards are generated. In turn, both the ethanol produced and the by-products obtained could be reused in the same temporary workplace.

Taking into consideration that the work area is characterized by its high yield per hectare of carrot, we conclude that the ethanol yield per hectare of cultivation is 3,100 L, making it comparable to the commonly employed energy crops for the production of ethanol presented in Table 2.

Conclusions

An integrated and sustainable fermentation process was designed to produce second-generation ethanol, revaluing two regional agro-industrial discards as well as by-product reuse. Discarded carrots and brewer's yeast, which are commonly used for animal feed, were processed to obtain 77.5 L of bioethanol, 450 kg of bagasse, and 970 L of vinasse per ton of discarded carrot. Bagasse was suitable for feeding 55 animals (calfs). Vinasse, after dilution with fresh water (1:5), satisfied the requirements necessary for beverage of the same number of animals, leaving a remnant which could be newly diluted (1:5) and used to irrigate a 0.025-ha carrot crop, the dimension of land required to grow 1 ton of carrot.

Abbreviations

CM: Carrot must; DC: Discarded carrot; RS: Reducing sugars; TS: Total sugars.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

NRA conceived the study, participated in its design, coordinated the tasks, helped in carrying out fermentations, and drafted the manuscript. ALC carried out the chemical analysis and interpretation of data and performed the statistical analysis. AC carried out the fermentations and interpretation of results and performed the statistical analysis. MLR participated in the design of the study and revised the results and manuscript critically for important intellectual content. JCY conceived the study, participated in its design, and helped draft the manuscript. All authors read and approved the final manuscript.

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